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NUCLEIC ACIDS ENCODING DEFENSE
INDUCIBLE PROTEINS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/272,227 filed February 28, 2001.

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FIELD OF THE INVENTION

The present invention relates generally to plant molecular biology. More specifically, it relates to nucleic acids and methods for modulating their expression in plants.

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BACKGROUND OF THE INVENTION

Plant disease outbreaks have resulted in catastrophic crop failures that have triggered famines and caused major social change. Generally, the best strategy for plant disease control is to use resistant cultivars selected or developed by plant breeders for this purpose. However, the potential for serious crop disease epidemics persists today, as evidenced by outbreaks of the Victoria blight of oats and southern corn leaf blight. Naturally occurring genetic resistance is often incomplete or race-specific and can be overcome by the evolution of new pathogens. Other options for treatment of plant disease are the application of chemicals. Unfortunately, chemical treatments are costly, sometimes difficult to apply effectively, and carry undesirable environmental risk. Accordingly, molecular methods are needed to supplement traditional breeding methods and chemical treatments to protect plants from pathogen attack.

Various genetic engineering strategies are being put forth to create enhanced disease resistance using recombinant DNA technology and transgenic plants. These genetic engineering strategies are meeting with varied success. No one strategy or

gene has proven to be a panacea, although some show promise. Successful broad improvement of crop resistance will likely require multiple strategies.

What is needed in the art is a method that overcomes the limitations of conventional breeding methods and existing genetic engineering strategies by providing a
5 discrete novel gene encoding an antimicrobial/antifungal protein that can be used in genetic engineering of plants to achieve enhanced resistance. The present invention provides this and other advantages.

BRIEF SUMMARY OF THE INVENTION

10 Generally, it is the object of the present invention to provide nucleic acids and proteins relating to a set of disease or stress inducible protein which are called AFP1. It is an object of the present invention to provide transgenic plants comprising the nucleic acids of the present invention. It is another object of the present invention to provide methods for modulating, in a transgenic plant, the expression of the nucleic acids of the
15 present invention. Another object of the present invention is to provide promoters capable of driving expression in a constitutive manner.

Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising a member selected from the group consisting of (a) a polynucleotide encoding a polypeptide of the present invention; (b) a polynucleotide amplified from a *Zea mays* nucleic acid library using the primers of the present invention; (c) a polynucleotide comprising at least 25 contiguous bases of the polynucleotides of the present invention;
20 (d) a polynucleotide encoding a maize AFP1 protein; (e) a polynucleotide having at least 80% sequence identity to the polynucleotides of the present invention; (f) a polynucleotide comprising at least 25 nucleotide in length which hybridizes under low stringency conditions to the polynucleotides of the present invention; (g) a polynucleotide comprising the sequence set forth in SEQ ID NOS: 1, 3, 5, 7, 9, 13, 15, 17, 19, 21, and
25 23; and (h) a polynucleotide complementary to a polynucleotide of (a) through (g). The isolated nucleic acid can be DNA. The isolated nucleic acid can also be RNA.

In another aspect, the present invention relates to vectors comprising the
30 polynucleotides of the present invention. Also the present invention relates to

recombinant expression cassettes, comprising a nucleic acid of the present invention operably linked to a promoter.

In another aspect, the present invention is directed to a host cell into which has been introduced the recombinant expression cassette.

5 In yet another aspect, the present invention relates to a transgenic plant or plant cell comprising a recombinant expression cassette with a promoter operably linked to any of the isolated nucleic acids of the present invention. Plants containing the recombinant expression cassette of the present invention include but are not limited to maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, and millet. The present
10 invention also provides transgenic seed from the transgenic plant.

In another aspect, the present invention relates to an isolated protein selected from the group consisting of (a) a polypeptide comprising at least 25 contiguous amino acids of an AFP1 protein; (b) a polypeptide which is a maize AFP1 protein; (c) a polypeptide comprising at least 75% sequence identity to a maize AFP1 protein; (d) a polypeptide
15 encoded by a nucleic acid of the present invention; and (e) a polypeptide characterized by SEQ ID NO: 2 and 4.

In further aspect, the present invention relates to a method of modulating the level of protein in a plant by introducing into a plant cell a recombinant expression cassette comprising a polynucleotide of the present invention operably linked to a promoter;
20 culturing the plant cell under plant growing conditions to produce a regenerated plant; and inducing expression of the polynucleotide for a time sufficient to modulate the protein of the present invention in the plant. Plants of the present invention include but are not limited to maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, and millet. The level of protein in the plant can either be increased or
25 decreased.

Definitions

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

30 Numeric ranges are inclusive of the numbers defining the range and include each integer within the defined range. Amino acids may be referred to herein by either their

commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The terms defined below are more fully defined by reference to the specification as a whole.

- 5 By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta
- 10 Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology: Principles and Applications*, D. H. Persing *et al.*, Ed., American Society for Microbiology, Washington, D.C. (1993). The product of amplification is termed an amplicon.
- 15 The term "antibody" includes reference to antigen binding forms of antibodies (e.g., Fab, F(ab)₂). The term "antibody" frequently refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bispecific antibodies).
- 20 The term "antigen" includes reference to a substance to which an antibody can be generated and/or to which the antibody is specifically immunoreactive. The specific immunoreactive sites within the antigen are known as epitopes or antigenic determinants. These epitopes can be a linear array of monomers in a polymeric composition - such as amino acids in a protein - or consist of or comprise a more complex secondary or tertiary structure. Those of skill will recognize that all immunogens (i.e., substances capable of
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- 30

eliciting an immune response) are antigens; however some antigens, such as haptens, are not immunogens but may be made immunogenic by coupling to a carrier molecule. An antibody immunologically reactive with a particular antigen can be generated *in vivo* or by recombinant methods such as selection of libraries of recombinant antibodies in phage 5 or similar vectors. See, e.g., Huse *et al.*, *Science* 246: 1275-1281 (1989); and Ward, *et al.*, *Nature* 341: 544-546 (1989); and Vaughan *et al.*, *Nature Biotech.* 14: 309-314 (1996).

As used herein, "antisense orientation" includes reference to a duplex 10 polynucleotide sequence, which is operably linked to a promoter in an orientation where the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited.

As used herein, "chromosomal region" includes reference to a length of a 15 chromosome, which may be measured, by reference to the linear segment of DNA, which it comprises. The chromosomal region can be defined by reference to two unique DNA sequences, i.e., markers.

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or conservatively 20 modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. 25 Such nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein which encodes a polypeptide also, by reference to the genetic code, describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; and UGG , 30 which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a

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polypeptide of the present invention is implicit in each described polypeptide sequence and is within the scope of the present invention.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the native protein for its native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

Alanine (A), Serine (S), Threonine (T);
Aspartic acid (D), Glutamic acid (E);
20 Asparagine (N), Glutamine (Q);
Arginine (R), Lysine (K);
Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) Proteins W.H. Freeman and Company.
By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as are

present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum*, or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed therein.

When the nucleic acid is prepared or altered synthetically, advantage can be taken
5 of known codon preferences of the intended host where the nucleic acid is to be
expressed. For example, although nucleic acid sequences of the present invention may be
expressed in both monocotyledonous and dicotyledonous plant species, sequences can be
modified to account for the specific codon preferences and GC content preferences of
monocotyledons or dicotyledons as these preferences have been shown to differ (Murray
10 *et al. Nucl. Acids Res.* 17: 477-498 (1989)). Thus, the maize preferred codon for a
particular amino acid may be derived from known gene sequences from maize. Maize
codon usage for 28 genes from maize plants are listed in Table 4 of Murray *et al.*, *supra*.

As used herein "full-length sequence" in reference to a specified polynucleotide
or its encoded protein means having the entire amino acid sequence of, a native (non-
synthetic), endogenous, biologically active form of the specified protein. Methods to
determine whether a sequence is full-length are well known in the art including such
exemplary techniques as northern or western blots, primer extension, S1 protection, and
ribonuclease protection. See, e.g., *Plant Molecular Biology: A Laboratory Manual*,
Clark, Ed., Springer-Verlag, Berlin (1997). Comparison to known full-length
20 homologous (orthologous and/or paralogous) sequences can also be used to identify full-
length sequences of the present invention. Additionally, consensus sequences typically
present at the 5' and 3' untranslated regions of mRNA aid in the identification of a
polynucleotide as full-length. For example, the consensus sequence ANNNAUGG,
where the underlined codon represents the N-terminal methionine, aids in determining
25 whether the polynucleotide has a complete 5' end. Consensus sequences at the 3' end,
such as polyadenylation sequences, aid in determining whether the polynucleotide has a
complete 3' end.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that
originates from a foreign species, or, if from the same species, is substantially modified
30 from its native form in composition and/or genomic locus by deliberate human
intervention. For example, a promoter operably linked to a heterologous structural gene

is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

5 By "host cell" is meant a cell, which contains a vector and supports the replication and/or expression of the vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells. One monocotyledonous host cell is a maize host cell.

10 The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the 15 incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

The term "isolated" refers to material, such as a nucleic acid or a protein, which 20 is: (1) substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment; or (2) if the material is in its natural environment, the material has been synthetically (non-naturally) altered by deliberate human intervention to a composition and/or placed at a 25 location in the cell (e.g., genome or subcellular organelle) not native to a material found in that environment. The alteration to yield the synthetic material can be performed on the material within or removed from its natural state. For example, a naturally occurring nucleic acid becomes an isolated nucleic acid if it is altered, or if it is transcribed from DNA which has been altered, by means of human intervention performed within the cell 30 from which it originates. See, e.g., Compounds and Methods for Site Directed Mutagenesis in Eukaryotic Cells, Kmiec, U.S. Patent No. 5,565,350; *In Vivo*

Homologous Sequence Targeting in Eukaryotic Cells; Zarling *et al.*, PCT/US93/03868.

Likewise, a naturally occurring nucleic acid (e.g., a promoter) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome not native to that nucleic acid. Nucleic acids which are "isolated" as defined herein, are also referred to as "heterologous" nucleic acids.

Unless otherwise stated, the term "AFP1 nucleic acid" is a nucleic acid of the present invention and means a nucleic acid comprising a polynucleotide of the present invention (a "AFP1 polynucleotide") encoding a AFP1 polypeptide. A "AFP1 gene" is a gene of the present invention and refers to a heterologous genomic form of a full-length AFP1 polynucleotide.

As used herein, "localized within the chromosomal region defined by and including" with respect to particular markers includes reference to a contiguous length of a chromosome delimited by and including the stated markers.

As used herein, "marker" includes reference to a locus on a chromosome that serves to identify a unique position on the chromosome. A "polymorphic marker" includes reference to a marker which appears in multiple forms (alleles) such that different forms of the marker, when they are present in a homologous pair, allow transmission of each of the chromosomes of that pair to be followed. A genotype may be defined by use of one or a plurality of markers.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., Vol. 1-3 (1989); and *Current*

Protocols in Molecular Biology, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994).

As used herein "operably linked" includes reference to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. One such plant is *Zea mays*.

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of

polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including among other things, simple and complex cells.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids.

- 5 The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they
- 10 15 may be circular, with or without branching, generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Further, this invention contemplates the use of both the methionine-containing and the
- 20 methionine-less amino terminal variants of the protein of the invention.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells whether nor not its origin is a plant cell. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells such *Agrobacterium* or *Rhizobium*. Examples of promoters under specific control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, or seeds. Such promoters are referred to as "tissue preferred". Promoters, which initiate transcription only in certain tissue, are referred to as "tissue specific". A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example,

vascular cells in roots or leaves. A "developmental" promoter is a promoter that initiates transcription at a specific time in the development of a plant, such as, at the time of flowering or seed set. An "inducible" or "repressible" promoter is a promoter, which is under environmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light. Tissue specific, tissue preferred, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter, which is active under most environmental conditions.

The term "AFP1 polypeptide" is a polypeptide of the present invention and refers to one or more amino acid sequences, in glycosylated or non-glycosylated form. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof. An "AFP1 protein" is a protein of the present invention and comprises an AFP1 polypeptide.

As used herein "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under-expressed or not expressed at all as a result of deliberate human intervention. The term "recombinant" as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a host cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

The term "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein,

polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass non-natural analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

- 5 The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least
10 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

The terms "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background).

- 15 Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected
20 (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash

in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, *Anal. Biochem.*, 138:267-284 (1984): $T_m = 81.5^{\circ}\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1 °C for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with ≥90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays",

Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

As used herein, "transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. Generally, the heterologous 5 polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those 10 transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial 15 transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

The following terms are used to describe the sequence relationships between two 20 or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

As used herein, "reference sequence" is a defined sequence used as a basis for 25 sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

As used herein, "comparison window" means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the 30 polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or

deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty

5 is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, *et al.*, *Nucleic Acids Research* 16: 10881-90 (1988); Huang, *et al.*, *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, *et al.*, *Methods in Molecular Biology* 24: 307-331 (1994). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

GAP uses the algorithm of Needleman and Wunsch (*J Mol Biol* 48: 443-453 (1970)) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension

penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the over the length of the gap times the gap extension penalty. Default gap creation penalty values and gap
5 extension penalty values in Version 10 of the Wisconsin Genetics Software Package are 8 and 2, respectively, for protein sequences. For nucleotide sequences, the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 100. Thus, for example, the gap creation and gap extension
10 penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the
15 number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software
20 Package is BLOSUM62 (see Henikoff and Henikoff, *Proc Natl Acad Sci USA* 89:10915).

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters. Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997) or GAP version 10 of Wisconsin Genetic Software Package using default parameters. Software for performing BLAST
25 analyses is publicly available, e.g., through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the
30 neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The

word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X 5 determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) *Proc. 10 Natl. Acad. Sci. USA* 89: 10915). In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 15 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match 20 probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance.

BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences, which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins although 25 other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, *Comput. Chem.*, 17:149-163 (1993)) and XNU (Claverie and States, *Comput. Chem.*, 17:191-201 (1993)) low-complexity filters can be employed alone or in combination.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences,

which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other 5 amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences, which differ by such conservative substitutions, are said to have "sequence similarity" or 10 "similarity". Means for making this adjustment are well known to those of skill in the art. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1.

15 The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the 20 portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of 25 matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably 30 at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard

parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. However, nucleic acids, which do not hybridize to each other under stringent conditions, are still substantially identical if the polypeptides which they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide, which the first nucleic acid encodes, is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

15 (ii) The terms "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Optionally, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides, which are "substantially similar" share sequences as, noted above except that 20 residue positions, which are not identical, may differ by conservative amino acid changes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention now will be described more fully hereinafter. This 30 invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are

provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the invention will come to mind to one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

Overview

The present invention provides, among other things, compositions and methods for modulating (i.e., increasing or decreasing) the level of polypeptides of the present invention in plants. In particular, the polypeptides of the present invention can be expressed at developmental stages, in tissues, and/or in quantities, which are uncharacteristic of non-recombinantly engineered plants. Thus, the present invention provides compositions useful in such exemplary applications as enhancing disease resistance. These genes encode a class of disease or stress inducible proteins. The compositions of the present invention can be used for enhancing disease resistance of crop plants, particularly those of the family Gramineae. The expression or modification of expression of these peptides, either constitutively, or in chosen tissues, or in response to pathogen attack, will enhance resistance in the plant to a pathogen.

By "disease resistance" is intended that the plants avoid the disease symptoms that are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen is minimized or lessened.

By "antipathogenic compositions" is intended that the compositions of the invention have antipathogenic activity and thus are capable of suppressing, controlling, and/or killing the invading pathogenic organism. An antipathogenic composition of the invention will reduce the disease symptoms resulting from pathogen challenge by at least about 5% to about 50%, at least about 10% to about 60%, at least about 30% to about

70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the invention can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

Assays that measure antipathogenic activity are commonly known in the art, as
5 are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. For example, a plant either expressing an antipathogenic polypeptide or having an antipathogenic composition
10 applied to its surface shows a decrease in tissue necrosis (*i.e.*, lesion diameter) or a decrease in plant death following pathogen challenge when compared to a control plant that was not exposed to the antipathogenic composition. Alternatively, antipathogenic activity can be measured by a decrease in pathogen biomass. For example, a plant expressing an antipathogenic polypeptide or exposed to an antipathogenic composition is
15 challenged with a pathogen of interest. Over time, tissue samples from the pathogen-inoculated tissues are obtained and RNA is extracted. The percent of a specific pathogen RNA transcript relative to the level of a plant specific transcript allows the level of pathogen biomass to be determined. See, for example, Thomma *et al.* (1998) *Plant Biology* 95:15107-15111, herein incorporated by reference.

20 Furthermore, *in vitro* antipathogenic assays include, for example, the addition of varying concentrations of the antipathogenic composition to paper disks and placing the disks on agar containing a suspension of the pathogen of interest. Following incubation, clear inhibition zones develop around the discs that contain an effective concentration of the antipathogenic polypeptide (Liu *et al.* (1994) *Plant Biology* 91:1888-1892, herein
25 incorporated by reference). Additionally, microspectrophotometrical analysis can be used to measure the *in vitro* antipathogenic properties of a composition (Hu *et al.* (1997) *Plant Mol. Biol.* 34:949-959 and Cammue *et al.* (1992) *J. Biol. Chem.* 267: 2228-2233, both of which are herein incorporated by reference). Pathogens of the invention are discussed below (see "Modulating Polypeptide Levels and/or Composition," below).

30 The present invention also provides isolated nucleic acid comprising polynucleotides of sufficient length and complementarity to a gene of the present

invention to use as probes or amplification primers in the detection, quantitation, or isolation of gene transcripts. For example, isolated nucleic acids of the present invention can be used as probes in detecting deficiencies in the level of mRNA in screenings for desired transgenic plants, for detecting mutations in the gene (e.g., substitutions, 5 deletions, or additions), for monitoring upregulation of expression or changes in enzyme activity in screening assays of compounds, for detection of any number of allelic variants (polymorphisms) of the gene, or for use as molecular markers in plant breeding programs. The isolated nucleic acids of the present invention can also be used for recombinant expression of their encoded polypeptides, or for use as immunogens in the 10 preparation and/or screening of antibodies. The isolated nucleic acids of the present invention can also be employed for use in sense or antisense suppression of one or more genes of the present invention in a host cell, tissue, or plant. Attachment of chemical agents which bind, intercalate, cleave and/or crosslink to the isolated nucleic acids of the present invention can also be used to modulate transcription or translation.

15 The present invention also provides isolated proteins comprising a polypeptide of the present invention (e.g., preproenzyme, proenzyme, or enzymes). The present invention also provides proteins comprising at least one epitope from a polypeptide of the present invention. The proteins of the present invention can be employed in assays for enzyme agonists or antagonists of enzyme function, or for use as immunogens or antigens 20 to obtain antibodies specifically immunoreactive with a protein of the present invention. Such antibodies can be used in assays for expression levels, for identifying and/or isolating nucleic acids of the present invention from expression libraries, or for purification of polypeptides of the present invention.

Thus, the expression of the molecules of the invention can be monitored, for 25 instance, to detect a disease state. Additionally, disease resistant plants for use in a breeding program can be selected based on constitutive expression of the AFP1 genes. That is, phenotypically normal plants that constitutively express AFP1 can be utilized. Progeny are screened for either resistance to a pathogen of interest or for the expression 30 of AFP1. Such plants have utility in breeding crop plants with constitutive, hereditary disease resistance.

The isolated nucleic acids and proteins of the present invention can be used over a broad range of plant types, particularly monocots such as the species of the family *Gramineae* including *Sorghum bicolor* and *Zea mays*. The isolated nucleic acid and proteins of the present invention can also be used in species from the genera: *Cucurbita*,
5 *Rosa*, *Vitis*, *Juglans*, *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*,
Vigna, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*,
Sinapis, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*,
Petunia, *Digitalis*, *Majorana*, *Ciahorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*,
Antirrhinum, *Heterocallis*, *Nemesis*, *Pelargonium*, *Panieum*, *Pennisetum*, *Ranunculus*,
10 *Senecio*, *Salpiglossis*, *Cucumis*, *Browalia*, *Glycine*, *Pisum*, *Phaseolus*, *Lolium*, *Oryza*,
Avena, *Hordeum*, *Secale*, and *Triticum*.

Nucleic Acids

The present invention provides, among other things, isolated nucleic acids of
15 RNA, DNA, and analogs and/or chimeras thereof, comprising a polynucleotide of the
present invention.

A polynucleotide of the present invention is inclusive of:

a polynucleotide encoding a polypeptide of SEQ ID NOS: SEQ ID NOS: 2, 4, 6,
8, 10, 14, 16, 18, 20, 22, 24 and conservatively modified and polymorphic variants
20 thereof, including exemplary polynucleotides of SEQ ID NOS: 1, 3, 5, 7, 9, 13, 15, 17,
19, 21, 23;

a polynucleotide which is the product of amplification from a *Zea mays* nucleic
acid library using primer pairs which selectively hybridize under stringent conditions to
loci within a polynucleotide selected from the group consisting of SEQ ID NOS: 1, 3, 5,
25 7, 9, 13, 15, 17, 19, 21, 23, wherein the polynucleotide has substantial sequence identity
to a polynucleotide selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 13,
15, 17, 19, 21, 23;

a polynucleotide which selectively hybridizes to a polynucleotide of (a) or (b);
a polynucleotide having a specified sequence identity with polynucleotides of (a),
30 (b), or (c);
complementary sequences of polynucleotides of (a), (b), (c), or (d); and

a polynucleotide comprising at least a specific number of contiguous nucleotides from a polynucleotide of (a), (b), (c), (d), or (e).

A. Polynucleotides Encoding A Polypeptide of the Present Invention or Conservatively Modified or Polymorphic Variants Thereof

5 The present invention provides isolated nucleic acids comprising a polynucleotide of the present invention, wherein the polynucleotide encodes a polypeptide of the present invention, or conservatively modified or polymorphic variants thereof. Those of skill in the art will recognize that the degeneracy of the genetic code allows for a plurality of polynucleotides to encode for the identical amino acid sequence. Such "silent variations" 10 can be used, for example, to selectively hybridize and detect allelic variants of polynucleotides of the present invention. Accordingly, the present invention includes polynucleotides of SEQ ID NOS: 1, 3, 5, 7, 9, 13, 15, 17, 19, 21, 23, and silent variations of polynucleotides encoding a polypeptide of SEQ ID NOS: 2, 4, 6, 8, 10, 14, 16, 18, 20, 22, 24. The present invention further provides isolated nucleic acids comprising 15 polynucleotides encoding conservatively modified variants of a polypeptide of SEQ ID NOS: 2, 4, 6, 8, 10, 14, 16, 18, 20, 22, 24. Conservatively modified variants can be used to generate or select antibodies immunoreactive to the non-variant polypeptide. Additionally, the present invention further provides isolated nucleic acids comprising polynucleotides encoding one or more polymorphic (allelic) variants of 20 polypeptides/polynucleotides. Polymorphic variants are frequently used to follow segregation of chromosomal regions in, for example, marker assisted selection methods for crop improvement.

B. Polynucleotides Amplified from a Zea mays Nucleic Acid Library

The present invention provides an isolated nucleic acid comprising a 25 polynucleotide of the present invention, wherein the polynucleotides are amplified from a *Zea mays* nucleic acid library. *Zea mays* lines B73, PHRE1, A632, BMS-P2#10, W23, and Mo17 are known and publicly available. Other publicly known and available maize lines can be obtained from the Maize Genetics Cooperation (Urbana, IL). The nucleic acid library may be a cDNA library, a genomic library, or a library generally constructed 30 from nuclear transcripts at any stage of intron processing. cDNA libraries can be normalized to increase the representation of relatively rare cDNAs. In optional

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embodiments, the cDNA library is constructed using a full-length cDNA synthesis method. Examples of such methods include Oligo-Capping (Maruyama, K. and Sugano, S. *Gene* 138: 171-174, 1994), Biotinylated CAP Trapper (Carninci, P., Kvan, C., et al. *Genomics* 37: 327-336, 1996), and CAP Retention Procedure (Edery, E., Chu, L.L., et al. *Molecular and Cellular Biology* 15: 3363-3371, 1995). cDNA synthesis is often catalyzed at 50-55°C to prevent formation of RNA secondary structure. Examples of reverse transcriptases that are relatively stable at these temperatures are SuperScript II Reverse Transcriptase (Life Technologies, Inc.), AMV Reverse Transcriptase (Boehringer Mannheim) and RetroAmp Reverse Transcriptase (Epicentre). Rapidly growing tissues, or rapidly dividing cells are preferably used as mRNA sources. Pathogen-infected leaf or seedling tissues are preferably used as mRNA sources. Exemplary crops for mRNA isolation include, but are not limited to, maize, rice or wheat.

The present invention also provides subsequences of the polynucleotides of the present invention. A variety of subsequences can be obtained using primers which selectively hybridize under stringent conditions to at least two sites within a polynucleotide of the present invention, or to two sites within the nucleic acid which flank and comprise a polynucleotide of the present invention, or to a site within a polynucleotide of the present invention and a site within the nucleic acid which comprises it. Primers are chosen to selectively hybridize, under stringent hybridization conditions, to a polynucleotide of the present invention. Generally, the primers are complementary to a subsequence of the target nucleic acid which they amplify. As those skilled in the art will appreciate, the sites to which the primer pairs will selectively hybridize are chosen such that a single contiguous nucleic acid can be formed under the desired amplification conditions. In optional embodiments, the primers will be constructed so that they selectively hybridize under stringent conditions to a sequence (or its complement) within the target nucleic acid which comprises the codon encoding the carboxy or amino terminal amino acid residue (i.e., the 3' terminal coding region and 5' terminal coding region, respectively) of the polynucleotides of the present invention. Optionally within these embodiments, the primers will be constructed to selectively hybridize entirely within the coding region of the target polynucleotide of the present invention such that the product of amplification of a cDNA target will consist of the

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coding region of that cDNA. The primer length in nucleotides is selected from the group of integers consisting of from at least 15 to 50. Thus, the primers can be at least 15, 18, 20, 25, 30, 40, or 50 nucleotides in length. Those of skill will recognize that a lengthened primer sequence can be employed to increase specificity of binding (i.e., annealing) to a target sequence. A non-annealing sequence at the 5' end of a primer (a "tail") can be added, for example, to introduce a cloning site at the terminal ends of the amplicon. Exemplary primer sequences include those of SEQ ID NOS: 11, 12.

The amplification products can be translated using expression systems well known to those of skill in the art and as discussed, *infra*. The resulting translation products can be confirmed as polypeptides of the present invention by, for example, assaying for the appropriate catalytic activity (e.g., specific activity and/or substrate specificity), or verifying the presence of one or more linear epitopes which are specific to a polypeptide of the present invention. Methods for protein synthesis from PCR derived templates are known in the art and available commercially. See, e.g., Amersham Life Sciences, Inc, Catalog '97, p.354.

Methods for obtaining 5' and/or 3' ends of a vector insert are well known in the art. See, e.g., RACE (Rapid Amplification of Complementary Ends) as described in Frohman, M. A., in PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, Inc., San Diego, 1990), pp. 28-38.); see also, U.S. Pat. No. 5,470,722, and *Current Protocols in Molecular Biology*, Unit 15.6, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); Frohman and Martin, *Techniques* 1:165 (1989).

C. Polynucleotides Which Selectively Hybridize to a Polynucleotide of (A) or
25 (B)

The present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides selectively hybridize, under selective hybridization conditions, to a polynucleotide of paragraphs (A) or (B) as discussed, *supra*. Thus, the polynucleotides of this embodiment can be used for isolating, 30 detecting, and/or quantifying nucleic acids comprising the polynucleotides of (A) or (B). For example, polynucleotides of the present invention can be used to identify, isolate, or

amplify partial or full-length clones in a deposited library. In some embodiments, the polynucleotides are genomic or cDNA sequences isolated or otherwise complementary to a cDNA from a dicot or monocot nucleic acid library. Exemplary species of monocots and dicots include, but are not limited to: maize, canola, soybean, cotton, wheat,
5 sorghum, sunflower, oats, sugar cane, millet, barley, and rice. Preferably, the cDNA library comprises at least 80% full-length sequences, preferably at least 85% or 90% full-length sequences, and more preferably at least 95% full-length sequences. The cDNA libraries can be normalized to increase the representation of rare sequences. Low stringency hybridization conditions are typically, but not exclusively, employed with
10 sequences having a reduced sequence identity relative to complementary sequences. Moderate and high stringency conditions can optionally be employed for sequences of greater identity. Low stringency conditions allow selective hybridization of sequences having about 70% sequence identity and can be employed to identify orthologous or paralogous sequences.

15

D. Polynucleotides Having a Specific Sequence Identity with the Polynucleotides of (A), (B) or (C)

The present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides have a specified identity at the
20 nucleotide level to a polynucleotide as disclosed above in paragraphs (A), (B), or (C). The percentage of identity to a reference sequence is at least 60% and, rounded upwards to the nearest integer, can be expressed as an integer selected from the group of integers consisting of from 60 to 99. Thus, for example, the percentage of identity to a reference sequence can be at least 70%, 75%, 80%, 85%, 90%, or 95%.

25

E. Polynucleotides Encoding a Protein Having a Subsequence from a Prototype Polypeptide and Cross-Reactive to the Prototype Polypeptide

The present invention provides isolated nucleic acids comprising polynucleotides of the present invention, wherein the polynucleotides encode a protein having a
30 subsequence of contiguous amino acids from a prototype polypeptide of the present invention such as are provided in section (A), above. The subsequences of a nucleotide

sequence may encode protein fragments that retain the biological activity of the native protein and hence confer disease resistance activity. Alternatively, subsequences of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, subsequences of a nucleotide 5 sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

The length of contiguous amino acids from the prototype polypeptide is selected from the group of integers consisting of from at least 10 to the number of amino acids 10 within the prototype sequence. Thus, for example, the polynucleotide can encode a polypeptide having a biologically active subsequence having at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 contiguous amino acids from the prototype polypeptide. Further, the number of such subsequences encoded by a polynucleotide of the instant embodiment can be any integer selected from the group 15 consisting of from 1 to 20, such as 2, 3, 4, or 5. The subsequences can be separated by any integer of nucleotides from 1 to the number of nucleotides in the sequence such as at least 5, 10, 15, 25, 50, 100, or 200 nucleotides.

Thus, a subsequence of an AFP1 nucleotide sequence may encode a biologically active portion of an AFP1 protein, or it may be a fragment that can be used as a 20 hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an AFP1 protein can be prepared by isolating a portion of one of the AFP1 nucleotide sequences of the invention, expressing the encoded portion of the AFP1 protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the AFP1 protein. Nucleic acid molecules that are subsequences of an 25 AFP1 nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, or 300 nucleotides, or up to the number of nucleotides present in a full-length AFP1 nucleotide sequence disclosed herein (for example, 676 nucleotides for SEQ ID NO:1, 574 nucleotides for SEQ ID NO:3, 577 nucleotides for SEQ ID NO:5, 580 nucleotides for SEQ ID NO:7, 529 nucleotides for SEQ ID NO:9, 348 nucleotides for SEQ ID NO:13, 30 591 nucleotides for SEQ ID NO:15, 524 nucleotides for SEQ ID NO:17, 584 nucleotides

for SEQ ID NO:19, 436 nucleotides for SEQ ID NO:21, or 584 nucleotides for SEQ ID NO:23.

The proteins encoded by polynucleotides of this embodiment, when presented as an immunogen, elicit the production of polyclonal antibodies which specifically bind to a prototype polypeptide such as (but not limited to) a polypeptide encoded by the polynucleotide of sections (A) or (B) above. Generally, however, a protein encoded by a polynucleotide of this embodiment does not bind to antisera raised against the prototype polypeptide when the antisera has been fully immunosorbed with the prototype polypeptide. Methods of making and assaying for antibody binding specificity/affinity are well known in the art. Exemplary immunoassay formats include ELISA, competitive immunoassays, radioimmunoassays, Western blots, indirect immunofluorescent assays and the like.

In one assay method, fully immunosorbed and pooled antisera that is elicited to the prototype polypeptide can be used in a competitive binding assay to test the protein. The concentration of the prototype polypeptide required to inhibit 50% of the binding of the antisera to the prototype polypeptide is determined. If the amount of the protein required to inhibit binding is less than twice the amount of the prototype protein, then the protein is said to specifically bind to the antisera elicited to the immunogen. Accordingly, the proteins of the present invention embrace allelic variants, conservatively modified variants, and minor recombinant modifications to a prototype polypeptide.

A polynucleotide of the present invention optionally encodes a protein having a molecular weight of the non-glycosylated protein within 20% of the molecular weight of the full-length non-glycosylated polypeptides of the present invention. Molecular weight can be readily determined by SDS-PAGE under reducing conditions. Optionally, the molecular weight is within 15% of a full-length polypeptide of the present invention, more preferably within 10% or 5%, and most preferably within 3%, 2%, or 1% of a full-length polypeptide of the present invention.

Optionally, the polynucleotides of this embodiment will encode a protein having a specific enzymatic activity at least 50%, 60%, 70%, 80%, or 90% of a cellular extract comprising the native, endogenous full-length polypeptide of the present invention. Further, the proteins encoded by polynucleotides of this embodiment will optionally have

a substantially similar affinity constant (K_m) and/or catalytic activity (*i.e.*, the microscopic rate constant, k_{cat}) as the native endogenous, full-length protein. Those of skill in the art will recognize that k_{cat}/K_m value determines the specificity for competing substrates and is often referred to as the specificity constant. Proteins of this embodiment
5 can have a k_{cat}/K_m value at least 10% of a full-length polypeptide of the present invention as determined using the endogenous substrate of that polypeptide. Optionally, the k_{cat}/K_m value will be at least 20%, 30%, 40%, 50%, and most preferably at least 60%, 70%, 80%, 90%, or 95% the k_{cat}/K_m value of the full-length polypeptide of the present invention.
Determination of k_{cat} , K_m , and k_{cat}/K_m can be determined by any number of means well
10 known to those of skill in the art. For example, the initial rates (*i.e.*, the first 5% or less of the reaction) can be determined using rapid mixing and sampling techniques (*e.g.*, continuous-flow, stopped-flow, or rapid quenching techniques), flash photolysis, or relaxation methods (*e.g.*, temperature jumps) in conjunction with such exemplary methods of measuring as spectrophotometry, spectrofluorimetry, nuclear magnetic
15 resonance, or radioactive procedures. Kinetic values are conveniently obtained using a Lineweaver-Burk or Eadie-Hofstee plot.

F. Polynucleotides Complementary to the Polynucleotides of (A)-(E)

The present invention provides isolated nucleic acids comprising polynucleotides
20 complementary to the polynucleotides of paragraphs A-D, above. As those of skill in the art will recognize, complementary sequences base-pair throughout the entirety of their length with the polynucleotides of (A)-(D) (*i.e.*, have 100% sequence identity over their entire length). Complementary bases associate through hydrogen bonding in double stranded nucleic acids. For example, the following base pairs are complementary:
25 guanine and cytosine; adenine and thymine; and adenine and uracil.

G. Polynucleotides that are Subsequences of the Polynucleotides of (A)-(F)

The present invention provides isolated nucleic acids comprising polynucleotides which comprise at least 15 contiguous bases from the polynucleotides of sections (A) (B),
30 (C), (D), (E), or (F) (*i.e.*, sections (A)-(F), as discussed above). A subsequence of an AFP1 nucleotide sequence may encode a biologically active portion of an AFP1 protein,

or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed elsewhere herein. Subsequences of an AFP1 nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of an AFP1 protein.

5 The length of the polynucleotide is given as an integer selected from the group consisting of from at least 15 to the length of the nucleic acid sequence from which the polynucleotide is a subsequence of. Thus, for example, polynucleotides of the present invention are inclusive of polynucleotides comprising at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 10 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 contiguous nucleotides in length from the polynucleotides of sections (A) through (F). Optionally, the number of such 15 subsequences encoded by a polynucleotide of the instant embodiment can be any integer selected from the group consisting of from 1 to 1000, such as 2, 3, 4, or 5. The subsequences can be separated by any integer of nucleotides from 1 to the number of nucleotides in the sequence such as at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides.

20 Subsequences can be made by *in vitro* synthetic, *in vitro* biosynthetic, or *in vivo* recombinant methods. In optional embodiments, subsequences can be made by nucleic acid amplification. For example, nucleic acid primers will be constructed to selectively hybridize to a sequence (or its complement) within, or co-extensive with, the coding region.

25 The subsequences of the present invention can comprise structural characteristics of the sequence from which it is derived. Alternatively, the subsequences can lack certain structural characteristics of the larger sequence from which it is derived such as a poly (A) tail. Optionally, a subsequence from a polynucleotide encoding a polypeptide having at least one linear epitope in common with a prototype polypeptide sequence as 30 provided in (a), above, may encode an epitope in common with the prototype sequence. Alternatively, the subsequence may not encode an epitope in common with the prototype

sequence but can be used to isolate the larger sequence by, for example, nucleic acid hybridization with the sequence from which it is derived. Subsequences can be used to modulate or detect gene expression by introducing into the subsequences compounds which bind, intercalate, cleave and/or crosslink to nucleic acids. Exemplary compounds 5 include acridine, psoralen, phenanthroline, naphthoquinone, daunomycin or chloroethylaminoaryl conjugates.

H. Polynucleotides that are Variants of the Polynucleotides of (A)-(G).

By "variants" is intended substantially similar sequences. For nucleotide 10 sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the AFP1 polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide 15 sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis, but which still encode a AFP1 protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 20 and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

I. Polynucleotides from a Full-length Enriched cDNA Library Having the 25 Physico-Chemical Property of Selectively Hybridizing to a Polynucleotide of (A)-(H)

The present invention provides an isolated polynucleotide from a full-length enriched cDNA library having the physico-chemical property of selectively hybridizing to a polynucleotide of sections (A), (B), (C), (D), (E), (F), (G), or (H) as discussed above. Methods of constructing full-length enriched cDNA libraries are known in the art and 30 discussed briefly below. The cDNA library comprises at least 50% to 95% full-length sequences (for example, at least 50%, 60%, 70%, 80%, 90%, or 95% full-length

sequences). The cDNA library can be constructed from a variety of tissues from a monocot or dicot at a variety of developmental stages. Exemplary species include maize, wheat, rice, canola, soybean, cotton, sorghum, sunflower, alfalfa, oats, sugar cane, millet, barley, and rice. Methods of selectively hybridizing, under selective hybridization conditions, a polynucleotide from a full-length enriched library to a polynucleotide of the present invention are known to those of ordinary skill in the art. Any number of stringency conditions can be employed to allow for selective hybridization. In optional embodiments, the stringency allows for selective hybridization of sequences having at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, up to 100% sequence identity over the length of the hybridized region. Full-length enriched cDNA libraries can be normalized to increase the representation of rare sequences.

J. Polynucleotide Products Made by a cDNA Isolation Process

The present invention provides an isolated polynucleotide made by the process of:

15 1) providing a full-length enriched nucleic acid library; and 2) selectively hybridizing the polynucleotide to a polynucleotide of sections (A), (B), (C), (D), (E), (F), (G), (H), or (I) as discussed above, and thereby isolating the polynucleotide from the nucleic acid library. Full-length enriched nucleic acid libraries are constructed and selective hybridization conditions are used, as discussed below. Such techniques, as well as nucleic acid 20 purification procedures, are well known in the art. Purification can be conveniently accomplished using solid-phase methods; such methods are well known to those of skill in the art and kits are available from commercial suppliers such as Advanced Biotechnologies (Surrey, UK). For example, a polynucleotide of sections (A)-(H) can be immobilized to a solid support such as a membrane, bead, or particle. See, e.g., U.S. 25 Patent No. 5,667,976. The polynucleotide product of the present process is selectively hybridized to an immobilized polynucleotide and the solid support is subsequently isolated from non-hybridized polynucleotides by methods including, but not limited to, centrifugation, magnetic separation, filtration, electrophoresis, and the like.

Construction of Nucleic Acids

The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified, or 5 otherwise constructed from a monocot. Embodiments include the monocot is *Zea mays*.

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the 10 isolation of the translated polynucleotide of the present invention. For example, a hexahistidine marker sequence provides a convenient means to purify the proteins of the present invention. A polynucleotide of the present invention can be attached to a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression 15 sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters, 20 and linkers is well known and extensively described in the art. For a description of various nucleic acids see, for example, Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA); and, Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, IL).

25 *A. Recombinant Methods for Constructing Nucleic Acids*

The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or a hybrid thereof, can be obtained from plant biological sources using any number of cloning methodologies known to those of skill in the art. In some embodiments, oligonucleotide probes which selectively hybridize, under stringent 30 conditions, to the polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library. While isolation of RNA, and construction

of cDNA and genomic libraries is well known to those of ordinary skill in the art, the following highlights some of the methods employed.

A1. mRNA Isolation and Purification

- 5 Total RNA from plant cells comprises such nucleic acids as mitochondrial RNA, chloroplastic RNA, rRNA, tRNA, hnRNA and mRNA. Total RNA preparation typically involves lysis of cells and removal of proteins, followed by precipitation of nucleic acids. Extraction of total RNA from plant cells can be accomplished by a variety of means. Frequently, extraction buffers include a strong detergent such as SDS and an organic
10 denaturant such as guanidinium isothiocyanate, guanidine hydrochloride or phenol. Following total RNA isolation, poly(A)⁺ mRNA is typically purified from the remainder RNA using oligo(dT) cellulose. Exemplary total RNA and mRNA isolation protocols are described in *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, et al.,
15 Eds., Greene Publishing and Wiley-Interscience, New York (1995). Total RNA and mRNA isolation kits are commercially available from vendors such as Stratagene (La Jolla, CA), Clonetech (Palo Alto, CA), Pharmacia (Piscataway, NJ), and 5'-3' (Paoli, PA). See also, U.S. Patent Nos. 5,614,391; and, 5,459,253. The mRNA can be fractionated into populations with size ranges of about 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 kb.
20 The cDNA synthesized for each of these fractions can be size selected to the same size range as its mRNA prior to vector insertion. This method helps eliminate truncated cDNA formed by incompletely reverse transcribed mRNA.

A2. Construction of a cDNA Library

- 25 Construction of a cDNA library generally entails five steps. First, first strand cDNA synthesis is initiated from a poly(A)⁺ mRNA template using a poly(dT) primer or random hexanucleotides. Second, the resultant RNA-DNA hybrid is converted into double stranded cDNA, typically by a combination of RNase H and DNA polymerase I (or Klenow fragment). Third, the termini of the double stranded cDNA are ligated to
30 adaptors. Ligation of the adaptors will produce cohesive ends for cloning. Fourth, size selection of the double stranded cDNA eliminates excess adaptors and primer fragments,

and eliminates partial cDNA molecules due to degradation of mRNAs or the failure of reverse transcriptase to synthesize complete first strands. Fifth, the cDNAs are ligated into cloning vectors and packaged. cDNA synthesis protocols are well known to the skilled artisan and are described in such standard references as: *Plant Molecular Biology*:

- 5 *A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). cDNA synthesis kits are available from a variety of commercial vendors such as Stratagene or Pharmacia.

A number of cDNA synthesis protocols have been described which provide
10 substantially pure full-length cDNA libraries. Substantially pure full-length cDNA
libraries are constructed to comprise at least 90%, and more preferably at least 93% or
95% full-length inserts amongst clones containing inserts. The length of insert in such
libraries can be from 0 to 8, 9, 10, 11, 12, 13, or more kilobase pairs. Vectors to
accommodate inserts of these sizes are known in the art and available commercially. See,
15 e.g., Stratagene's lambda ZAP Express (cDNA cloning vector with 0 to 12 kb cloning
capacity).

An exemplary method of constructing a greater than 95% pure full-length cDNA
library is described by Carninci et al., *Genomics*, 37:327-336 (1996). In that protocol,
the cap-structure of eukaryotic mRNA is chemically labeled with biotin. By using
20 streptavidin-coated magnetic beads, only the full-length first-strand cDNA/mRNA
hybrids are selectively recovered after RNase I treatment. The method provides a high
yield library with an unbiased representation of the starting mRNA population. Other
methods for producing full-length libraries are known in the art. See, e.g., Edery et al.,
Mol. Cell Biol., 15(6):3363-3371 (1995); and, PCT Application WO 96/34981.

25

A3. Normalized or Subtracted cDNA Libraries

A non-normalized cDNA library represents the mRNA population of the tissue it
was made from. Since unique clones are out-numbered by clones derived from highly
expressed genes their isolation can be laborious. Normalization of a cDNA library is the
30 process of creating a library in which each clone is more equally represented.

A number of approaches to normalize cDNA libraries are known in the art. One approach is based on hybridization to genomic DNA. The frequency of each hybridized cDNA in the resulting normalized library would be proportional to that of each corresponding gene in the genomic DNA. Another approach is based on kinetics. If
5 cDNA reannealing follows second-order kinetics, rarer species anneal less rapidly and the remaining single-stranded fraction of cDNA becomes progressively more normalized during the course of the hybridization. Specific loss of any species of cDNA, regardless of its abundance, does not occur at any Cot value. Construction of normalized libraries is described in Ko, *Nucl. Acids. Res.*, 18(19):5705-5711 (1990); Patanjali *et al.*, *Proc. Natl. Acad. U.S.A.*, 88:1943-1947 (1991); U.S. Patents 5,482,685, and 5,637,685. In an exemplary method described by Soares *et al.*, normalization resulted in reduction of the abundance of clones from a range of four orders of magnitude to a narrow range of only 1 order of magnitude. *Proc. Natl. Acad. Sci. USA*, 91:9228-9232 (1994).

Subtracted cDNA libraries are another means to increase the proportion of less abundant cDNA species. In this procedure, cDNA prepared from one pool of mRNA is depleted of sequences present in a second pool of mRNA by hybridization. The cDNA:mRNA hybrids are removed and the remaining un-hybridized cDNA pool is enriched for sequences unique to that pool. See, Foote *et al.* in, *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); Kho and Zarbl, *Technique*, 3(2):58-63 (1991); Sive and St. John, *Nucl. Acids Res.*, 16(22):10937 (1988); *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); and, Swaroop *et al.*, *Nucl. Acids Res.*, 19(8):1954 (1991). cDNA subtraction kits are commercially available. See, e.g., PCR-Select (Clontech).

25

A4. Construction of a Genomic Library

To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector.

30 Methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate molecular biological

techniques and instructions sufficient to direct persons of skill through many construction, cloning, and screening methodologies are found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Vols. 1-3 (1989), Methods in Enzymology, Vol. 152: *Guide to Molecular Cloning Techniques*, Berger and Kimmel, Eds., San Diego: Academic Press, Inc. (1987), *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997). Kits for construction of genomic libraries are also commercially available.

10

A5. Nucleic Acid Screening and Isolation Methods

The cDNA or genomic library can be screened using a probe based upon the sequence of a polynucleotide of the present invention such as those disclosed herein. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate 15 homologous genes in the same or different plant species. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and the target for duplex formation to occur. The 20 degree of stringency can be controlled by temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide. For example, the stringency of hybridization is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of formamide within the range of 0% to 50%. The degree of complementarity (sequence identity) required for detectable 25 binding will vary in accordance with the stringency of the hybridization medium and/or wash medium. The degree of complementarity will optimally be 100 percent; however, it should be understood that minor sequence variations in the probes and primers may be compensated for by reducing the stringency of the hybridization and/or wash medium.

The nucleic acids of interest can also be amplified from nucleic acid samples 30 using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of polynucleotides of the present

invention and related genes directly from genomic DNA or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, U.S. Patent No. 4,683,202 (1987); and, *PCR Protocols A Guide to Methods and Applications*, Innis *et al.*, Eds., Academic Press Inc., San Diego, CA (1990). Commercially available kits for genomic PCR amplification are known in the art. See, 5 e.g., Advantage-GC Genomic PCR Kit (Clontech). The T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

10 PCR-based screening methods have also been described. Wilfinger *et al.* describe a PCR-based method in which the longest cDNA is identified in the first step so that incomplete clones can be eliminated from study. *BioTechniques*, 22(3): 481-486 (1997). 15 In that method, a primer pair is synthesized with one primer annealing to the 5' end of the sense strand of the desired cDNA and the other primer to the vector. Clones are pooled to allow large-scale screening. By this procedure, the longest possible clone is identified amongst candidate clones. Further, the PCR product is used solely as a diagnostic for the presence of the desired cDNA and does not utilize the PCR product itself. Such methods 20 are particularly effective in combination with a full-length cDNA construction methodology, *supra*.

B. Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids of the present invention can also be prepared by direct 25 chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.* 22: 1859-1862 (1981); the solid phase phosphoramidite triester method 30 described by Beaucage and Caruthers, *Tetra. Letts.* 22(20): 1859-1862 (1981), e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter *et al.*, *Nucleic Acids Res.*, 12: 6159-6168 (1984); and, the solid support method of U.S. Patent No.

4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Recombinant Expression Cassettes

The present invention further provides recombinant expression cassettes comprising a nucleic acid of the present invention. A nucleic acid sequence coding for the desired polynucleotide of the present invention, for example a cDNA or a genomic sequence encoding a full length polypeptide of the present invention, can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

For example, plant expression vectors may include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

A number of promoters can be used in the practice of the invention. A plant promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the ubiquitin 1

promoter (Christensen, *et al.* *Plant Mol Biol* 18, 675-689 (1992); Bruce, *et al.*, *Proc Natl Acad Sci USA* 86, 9692-9696 (1989)), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the *Nos* promoter, the pEmu promoter, the rubisco promoter, the GRP1-8 promoter, and other transcription initiation regions from various plant genes known to those of skill. For constitutive expression of the polynucleotides of the present invention, the ubiquitin 1 promoter is the preferred promoter.

Where low level expression is desired, weak promoters will be used. It is recognized that weak inducible promoters may be used. Additionally, either a weak 10 constitutive or a weak tissue specific promoter may be used. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By low level is intended at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Alternatively, it is recognized that weak promoters also encompass promoters that are expressed in only a few cells and not in 15 others to give a total low level of expression. Such weak constitutive promoters include, for example, the core promoter of the Rsyn7 promoter (WO 97/44756), the core 35S CaMV promoter, and the like. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention under environmental control. Such promoters are referred to here as "inducible" promoters. Environmental conditions that may effect transcription by 20 inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. Examples of inducible promoters are the Adh1 promoter, which is inducible by hypoxia or cold stress, the Hsp70 promoter, which is inducible by heat stress, and the PPDK promoter, which is inducible by light. Examples of pathogen-inducible promoters include those from proteins, which are induced following infection by a pathogen; *e.g.*, PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi, *et al.*, *Neth J. Plant Pathol.* 89:245-254 (1983); Uknnes, *et al.*, *The Plant Cell* 4:645-656 (1992); Van Loon, *Plant Mol. Virol.* 4:111-116 (1985); copending U. S. application 25 30

number 60/076,100, filed February 26, 1998; and copending U. S. application number 60/079,648, filed March 27, 1998.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau, *et al.*, *Plant Mol Biol* 9:335-342 (1987); Matton, 5 *et al.*, *Molecular Plant-Microbe Interactions* 2:325-342 (1987); Somssich *et al.*, *Proc Natl Acad Sci USA* 83:2427-2430 (1986); Somssich *et al.*, *Mol Gen Genetics* 2:93-98 (1988); Yang, *Proc Natl Acad Sci USA* 93:14972-14977. See also, Chen, *et al.*, *Plant J* 10:955-966 (1996); Zhang and Sing, *Proc Natl Acad Sci USA* 91:2507-2511 (1994); Warner, *et al.*, *Plant J* 3:191-201 (1993); and Siebertz, *et al.*, *Plant Cell* 1:961-968 10 (1989), all of which are herein incorporated by reference. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero, *et al.*, *Physiol Molec Plant Path* 41:189-200 (1992) and is herein incorporated by reference.

Additionally, as pathogens find entry into plants through wounds or insect 15 damage, a wound inducible promoter may be used in the constructs of the invention. Such wound inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan, *Annu Rev Phytopath* 28:425-449 (1990); Duan, *et a.*, *Nat Biotech* 14:494-498 (1996)); wun1 and wun2, US Patent No. 5,428,148; win1 and win2 (Stanford, *et al.*, *Mol Gen Genet* 215:200-208 (1989)); systemin (McGurl, *et al.*, *Science* 225:1570-1573 (1992)); 20 WIP1 (Rohmeier, *et al.*, *Plant Mol Biol* 22:783-792 (1993); Eckelkamp, *et al.*, *FEB Letters* 323:73-76 (1993)); MPI gene (Corderok, *et al.*, *The Plant J* 6(2):141-150(1994)); and the like, herein incorporated by reference.

Examples of promoters under developmental control include promoters that 25 initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. Exemplary promoters include the anther specific promoter 5126 (U.S. Patent Nos. 5,689,049 and 5,689,051), glob-1 promoter, and gamma-zein promoter. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations. An inducible promoter can also be modified, if necessary, for weak expression.

Tissue-preferred promoters can be utilized to target enhanced AFP1 expression 30 within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.*

(1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

10 Leaf-specific promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

15 Both heterologous and non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the nucleic acids of the present invention. These promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter concentration and/or composition of the proteins of the present invention in a desired tissue. Thus, in some 20 embodiments, the nucleic acid construct will comprise a promoter functional in a plant cell, such as in *Zea mays*, operably linked to a polynucleotide of the present invention. Promoters useful in these embodiments include the endogenous promoters driving expression of a polypeptide of the present invention.

In some embodiments, isolated nucleic acids which serve as promoter or enhancer 25 elements can be introduced in the appropriate position (generally upstream) of a non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example, endogenous promoters can be altered *in vivo* by mutation, deletion, and/or substitution (see, Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868), or isolated 30 promoters can be introduced into a plant cell in the proper orientation and distance from a gene of the present invention so as to control the expression of the gene. Gene

expression can be modulated under conditions suitable for plant growth to alter the total concentration and/or alter the composition of the polypeptides of the present invention in plant cell. Thus, the present invention provides compositions, and methods for making, heterologous promoters and/or enhancers operably linked to a native, endogenous (i.e., non-heterologous) form of a polynucleotide of the present invention.

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene.

An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold. Buchman and Berg, *Mol. Cell Biol.* 8: 4395-4405 (1988); Callis *et al.*, *Genes Dev.* 1: 1183-1200 (1987). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1 intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994).

The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene, which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes including genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resistance to herbicides which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance in

particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

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Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, Meth. in Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, the vectors 10 integrate a portion of vector DNA into the genome of the host plant. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA).

15

A polynucleotide of the present invention can be expressed in either sense or anti-sense orientation as desired. It will be appreciated that control of gene expression in either sense or anti-sense orientation can have a direct impact on the observable plant characteristics. Antisense technology can be conveniently used to inhibit gene expression in plants. To accomplish this, a nucleic acid segment from the desired gene is cloned and 20 operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has been shown that antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy *et al.*, Proc. Nat'l. Acad. Sci. (USA) 85: 8805-8809 (1988); and Hiatt *et al.*, 25 U.S. Patent No. 4,801,340.

30

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli *et al.*, *The Plant Cell* 2: 279-289 (1990) and U.S. Patent No. 5,034,323.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of plant genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff *et al.*, *Nature* 334: 585-591 (1988).

A variety of cross-linking agents, alkylating agents and radical generating species as pendant groups on polynucleotides of the present invention can be used to bind, label, detect, and/or cleave nucleic acids. For example, Vlassov, V. V., *et al.*, *Nucleic Acids Res* (1986) 14:4065-4076, describe covalent bonding of a single-stranded DNA fragment with alkylating derivatives of nucleotides complementary to target sequences. A report of similar work by the same group is that by Knorre, D. G., *et al.*, *Biochimie* (1985) 67:785-789. Iverson and Dervan also showed sequence-specific cleavage of single-stranded DNA mediated by incorporation of a modified nucleotide which was capable of activating cleavage (*J Am Chem Soc* (1987) 109:1241-1243). Meyer, R. B., *et al.*, *J Am Chem Soc* (1989) 111:8517-8519, effect covalent crosslinking to a target nucleotide using an alkylating agent complementary to the single-stranded target nucleotide sequence. A photoactivated crosslinking to single-stranded oligonucleotides mediated by psoralen was disclosed by Lee, B. L., *et al.*, *Biochemistry* (1988) 27:3197-3203. Use of crosslinking in triple-helix forming probes was also disclosed by Home, *et al.*, *J Am Chem Soc* (1990) 112:2435-2437. Use of N4, N4-ethanocytosine as an alkylating agent to crosslink to single-stranded oligonucleotides has also been described by Webb and Matteucci, *J Am Chem Soc* (1986) 108:2764-2765; *Nucleic Acids Res* (1986) 14:7661-7674; Feteritz *et al.*, *J. Am. Chem. Soc.* 113:4000 (1991). Various compounds to bind, detect, label, and/or cleave nucleic acids are known in the art. See, for example, U.S. Patent Nos. 5,543,507; 5,672,593; 5,484,908; 5,256,648; and, 5,681,941.

Proteins

The isolated proteins of the present invention comprise a polypeptide having at least 10 amino acids encoded by any one of the polynucleotides of the present invention as discussed more fully, *supra*, or polypeptides which are conservatively modified

- 5 variants thereof. The proteins of the present invention or variants thereof can comprise any number of contiguous amino acid residues from a polypeptide of the present invention, wherein that number is selected from the group of integers consisting of from 10 to the number of residues in a full-length polypeptide of the present invention. Optionally, this subsequence of contiguous amino acids is at least 15, 16, 17, 18, 19, 20,
- 10 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, or 40 amino acids in length, often at least 50, 55, 60, 65, 70, 75, 80, 85, or 90 amino acids in length. Further, the number of such subsequences can be any integer selected from the group consisting of from 1 to 20, such as 2, 3, 4, or 5.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, disease resistance activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native AFP1 protein of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more 25 preferably at least about 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

30 As those of skill will appreciate, the present invention includes catalytically active polypeptides of the present invention (i.e., enzymes). Catalytically active polypeptides

have a specific activity of at least 20%, 30%, or 40%, and preferably at least 50%, 60%, or 70%, and most preferably at least 80%, 90%, or 95% that of the native (non-synthetic), endogenous polypeptide. Further, the substrate specificity (k_{cat}/K_m) is optionally substantially similar to the native (non-synthetic), endogenous polypeptide. Typically, 5 the K_m will be at least 30%, 40%, or 50%, that of the native (non-synthetic), endogenous polypeptide; and more preferably at least 60%, 70%, 80%, or 90%. Methods of assaying and quantifying measures of enzymatic activity and substrate specificity (k_{cat}/K_m), are well known to those of skill in the art.

Generally, the proteins of the present invention will, when presented as an 10 immunogen, elicit production of an antibody specifically reactive to a polypeptide of the present invention. Further, the proteins of the present invention will not bind to antisera raised against a polypeptide of the present invention which has been fully immunosorbed with the same polypeptide. Immunoassays for determining binding are well known to those of skill in the art. One immunoassay is a competitive immunoassay as discussed, 15 *infra*. Thus, the proteins of the present invention can be employed as immunogens for constructing antibodies immunoreactive to a protein of the present invention for such exemplary utilities as immunoassays or protein purification techniques.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are 20 generally known in the art. For example, amino acid sequence variants of the AFP1 proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in 25 Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one 30 amino acid with another having similar properties, may be preferable.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired disease resistance activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by disease resistance assays, see above.

As discussed elsewhere herein, variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different AFP1 coding sequences can be manipulated to create a new AFP1 possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*.

Expression of Proteins in Host Cells

Using the nucleic acids of the present invention, one may express a protein of the present invention in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. In one embodiment, proteins of the present invention are expressed in plant leaf tissues. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the

present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the 5 DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To 10 obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. One of skill would recognize that modifications can be made to a protein of the present invention without diminishing its biological activity. Some modifications 15 may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification 20 sequences.

A. Expression in Prokaryotes

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains 25 may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake et al., Nature 292:128 (1981)). The inclusion of selection markers in 30

DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell.

Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, et al., *Gene* 22: 229-235 (1983); Mosbach, et al., *Nature* 302: 543-545 (1983)).

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B. Expression in Eukaryotes

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. Sherman, F., et al., *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1982) is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeast for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

30 The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance,

mammalian, insect, or plant origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.*, *Immunol. Rev.* 89: 49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992).

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See Schneider, *J. Embryol. Exp. Morphol.* 27: 353-365 (1987)).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, *J. Virol.* 45: 773-781 (1983)). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector in *DNA Cloning Vol. II a Practical Approach*, D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238 (1985).

Transfection/Transformation of Cells

The method of transformation/transfection is not critical to the instant invention; various methods of transformation or transfection are currently available. As newer

methods are available to transform crops or other host cells they may be directly applied. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription and/or translation of the sequence to effect phenotypic changes in the organism. Thus, any method which provides for efficient transformation/transfection may be employed.

- The polynucleotides of the present invention can be used to transform any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols may vary depending on the type of plant cell, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *BioTechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.* U.S. Patent 4,945,050; Tomes *et al.* 10 "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995); and McCabe *et al.* (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment" In Gamborg and Phillips (Eds.) *Plant Cell, Tissue and Organ Culture: Fundamental Methods*, Springer-Verlag, Berlin (1995) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize) 15 Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooydaas-Van Slogteren & Hooykaas (1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) In *The Experimental Manipulation of Ovule Tissues* ed. G.P. Chapman *et al.* pp. 197-209. Longman, NY (pollen); Kaepller *et al.* (1990) *Plant Cell Reports* 9:415-418; and Kaepller *et al.* (1992) 20

Theor. Appl. Genet. 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); LI *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

5 The cells, which have been transformed, may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports*, 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure
10 that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved. One of skill will recognize that after the recombinant expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be
15 used, depending upon the species to be crossed.

 In vegetatively propagated crops, mature transgenic plants can be propagated by the taking of cuttings or by tissue culture techniques to produce multiple identical plants. Selection of desirable transgenics is made and new varieties are obtained and propagated vegetatively for commercial use. In seed propagated crops, mature transgenic plants can be self-crossed to produce a homozygous inbred plant. The inbred plant produces seed containing the newly introduced heterologous nucleic acid. These seeds can be grown to produce plants that would produce the selected phenotype.

 Parts obtained from the regenerated plant, such as flowers, seeds, leaves, branches, fruit, and the like are included in the invention, if these parts comprise cells comprising the isolated nucleic acid of the present invention. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, if these parts comprise the introduced nucleic acid sequences.

 An embodiment is a transgenic plant that is homozygous for the added heterologous nucleic acid; i.e., a transgenic plant that contains two added nucleic acid sequences, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) a

heterozygous transgenic plant that contains a single added heterologous nucleic acid, germinating some of the seed produced and analyzing the resulting plants produced for altered expression of a polynucleotide of the present invention relative to a control plant (i.e., native, non-transgenic). Backcrossing to a parental plant and out-crossing with a
5 non-transgenic plant are also contemplated.

Animal and lower eukaryotic (e.g., yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of
10 the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation, biolistics, and micro-injection of the DNA directly into the cells. The transfected cells are cultured by means well known in the art. Kuchler, R.J., *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc. (1977).

15 **Synthesis of Proteins**

The proteins of the present invention can be constructed using non-cellular synthetic methods. Solid phase synthesis of proteins of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the
20 sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, Solid-Phase Peptide Synthesis, pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology*. Vol. 2: *Special Methods in Peptide Synthesis, Part A.*; Merrifield, et al., *J. Am. Chem. Soc.* 85: 2149-2156 (1963), and Stewart et al., *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chem. Co., Rockford, Ill. (1984). Proteins of greater length may be synthesized
25 by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide)) is known to those of skill.

Purification of Proteins

30 The proteins of the present invention may be purified by standard techniques well known to those of skill in the art. Recombinantly produced proteins of the present

invention can be directly expressed or expressed as a fusion protein. The recombinant protein is purified by a combination of cell lysis (e.g., sonication, French press) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired recombinant protein.

- 5 The proteins of this invention, recombinant or synthetic, may be purified to substantial purity by standard techniques well known in the art, including detergent solubilization, selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. *See*, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982);
- 10 Deutscher, *Guide to Protein Purification*, Academic Press (1990). For example, antibodies may be raised to the proteins as described herein. Purification from *E. coli* can be achieved following procedures described in U.S. Patent No. 4,511,503. The protein may then be isolated from cells expressing the protein and further purified by standard protein chemistry techniques as described herein. Detection of the expressed protein is
- 15 achieved by methods known in the art and include, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation.

The AFP1 proteins of the invention can be used for any application including coating surfaces to target microbes. In this manner, the target microbes include human pathogens or microorganisms. Surfaces that might be coated with the AFP1 proteins of the invention include carpets and sterile medical facilities. Polymer bound polypeptides of the invention may be used to coat surfaces. Methods for incorporating compositions with antimicrobial properties into polymers are known in the art. See U.S. Patent No. 5,847,047, herein incorporated by reference.

25 **Modulating Polypeptide Levels and/or Composition**

The present invention further provides a method for modulating (i.e., increasing or decreasing) the concentration or composition of the polypeptides of the present invention in a plant or part thereof. Modulation can be effected by increasing or decreasing the concentration and/or the composition (i.e., the ratio of the polypeptides of the present invention) in a plant. The method comprises transforming a plant cell with a recombinant expression cassette comprising a polynucleotide of the present invention as

described above to obtain a transformed plant cell, growing the transformed plant cell under plant forming conditions, and inducing expression of a polynucleotide of the present invention in the plant for a time sufficient to modulate concentration and/or composition in the plant or plant part.

5 In some embodiments, the content and/or composition of polypeptides of the present invention in a plant may be modulated by altering, *in vivo* or *in vitro*, the promoter of a non-isolated gene of the present invention to up- or down-regulate gene expression. In some embodiments, the coding regions of native genes of the present invention can be altered via substitution, addition, insertion, or deletion to decrease
10 activity of the encoded enzyme. See, e.g., Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868. And in some embodiments, an isolated nucleic acid (e.g., a vector) comprising a promoter sequence is transfected into a plant cell. Subsequently, a plant cell comprising the promoter operably linked to a polynucleotide of the present invention is selected for by means known to those of skill in the art such as, but not limited to,
15 Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the gene and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or composition of polypeptides of the present invention in the plant. Plant forming conditions are well
20 known in the art and discussed briefly, *supra*.

In general, concentration or composition is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned recombinant expression cassette.
Modulation in the present invention may occur during and/or subsequent to growth of the
25 plant to the desired stage of development. Modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of the present invention in, for example, sense or antisense orientation as discussed in greater detail, *supra*. Induction of expression of a polynucleotide of the present invention can also be controlled by
30 exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds which activate expression from these promoters are

well known in the art. In one embodiments, the induction of expression of a polynucleotide of the present invention can also be modulated, relative to an untreated control, by infection with a pathogen such as viruses or viroids, bacteria, insects, fungi, and the like. Viruses include, but are not limited to, tobacco or cucumber mosaic virus, 5 ringspot virus, necrosis virus, and maize dwarf mosaic virus. Specific fungal and viral pathogens for the major crops include, but are not limited to: Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae* (*Phomopsis sojae*), *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolfsii*, *Cercospora kikuchii*, 10 *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium* (*Colletotichum truncatum*), *Corynespora cassiicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, Soybean mosaic virus, *Glomerella glycines*, Tobacco Ring spot virus, Tobacco Streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, Tomato 15 spotted wilt virus, *Heterodera glycines* *Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassiccola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibacter michiganense* subsp. 20 *insidiosum*, *Pythium ultimum*, *Pythium irregularare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrichila medicaginis*, *Fusarium avenaceum*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: 25 *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotrichum graminicola*, *Erysiphe graminis* f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*, *Puccinia striiformis*, *Pyrenophora triticeirepentis*, 30 *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercospora*

herpotrichoides, Rhizoctonia solani, Rhizoctonia cerealis, Gaeumannomyces graminis
var. *tritici, Pythium aphanidermatum, Pythium arrhenomannes, Pythium ultimum,*
Bipolaris sorokiniana, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil Borne
Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American
5 *Wheat Striate Virus, Claviceps purpurea, Tilletia tritici, Tilletia laevis, Ustilago tritici,*
Tilletia indica, Rhizoctonia solani, Pythium arrhenomannes, Pythium gramicola, Pythium
aphanidermatum, High Plains Virus, European wheat striate virus; Sunflower:
Plasmopora halstedii, Sclerotinia sclerotiorum, Aster Yellows, Septoria helianthi,
Phomopsis helianthi, Alternaria helianthi, Alternaria zinniae, Botrytis cinerea, Phoma
10 *macdonaldii, Macrohomina phaseolina, Erysiphe cichoracearum, Rhizopus oryzae,*
Rhizopus arrhizus, Rhizopus stolonifer, Puccinia helianthi, Verticillium dahliae, Erwinia
carotovorum p.v. Carotovora, Cephalosporium acremonium, Phytophthora cryptogea,
Albugo tragopogonis; Corn: Fusarium moniliforme var. subglutinans, Erwinia stewartii,
15 *Fusarium moniliforme, Gibberella zeae (Fusarium graminearum), Stenocarpella maydis*
(*Diplodia maydis*), *Pythium irregularare, Pythium debaryanum, Pythium graminicola,*
Pythium splendens, Pythium ultimum, Pythium aphanidermatum, Aspergillus flavus,
Bipolaris maydis O, T (Cochliobolus heterostrophus), Helminthosporium carbonum I, II
& III (*Cochliobolus carbonum*), *Exserohilum turcicum I, II & III, Helminthosporium*
pedicellatum, Physoderma maydis, Phyllosticta maydis, Kabatina-maydis, Cercospora
20 *sorghii, Ustilago maydis, Puccinia sorghi, Puccinia polysora, Macrohomina*
phaseolina, Penicillium oxalicum, Nigrospora oryzae, Cladosporium herbarum,
Curvularia lunata, Curvularia inaequalis, Curvularia pallens, Clavibacter
michiganense subsp. nebraskense, Trichoderma viride, Maize Dwarf Mosaic Virus A & B,
Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, Claviceps sorghi,
25 *Pseudonomas avenae, Erwinia chrysanthemi p.v. Zea, Erwinia corotovora, Cornstunt*
spiroplasma, Diplodia macrospora, Sclerotiora macrospora, Peronosclerospora
sorghi, Peronosclerospora philippinesis, Peronosclerospora maydis, Peronosclerospora
sacchari, Spacelotheca reiliana, Physopella zeae, Cephalosporium maydis,
Cephalosporium acremonium, Maize Chlorotic Mottle Virus, High Plains Virus, Maize
30 *Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus, Maize Stripe Virus, Maize*
Rough Dwarf Virus; Sorghum: Exserohilum turcicum, Colletotrichum graminicola

- (*Glomerella graminicola*), *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternate*, *Bipolaris sorghicola*,
- 5 *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae* (*Pseudomonas alboprecipitans*), *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum* (*Sphacelotheca reiliana*), *Sphacelotheca cruenta*, *Sporisorium sorghi*, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*, *Sclerotophthora macrospora*,
- 10 *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, and *Pythium graminicola*. In one embodiments, the polypeptides of the present invention are modulated in monocots, particularly maize, rice, or wheat.

15 **Molecular Markers**

The present invention provides a method of genotyping a plant comprising a polynucleotide of the present invention. Preferably, the plant is a monocot, such as maize or sorghum. Genotyping provides a means of distinguishing homologs of a chromosome pair and can be used to differentiate segregants in a plant population. Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, *The DNA Revolution* by Andrew H. Paterson 1996 (Chapter 2) in: *Genome Mapping in Plants* (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp.7-21.

The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). RFLPs are the product of allelic differences between DNA restriction fragments caused by nucleotide sequence variability. As is well known to those of skill in the art, RFLPs are typically detected by extraction of genomic

DNA and digestion with a restriction enzyme. Generally, the resulting fragments are separated according to size and hybridized with a probe; single copy probes are preferred. Restriction fragments from homologous chromosomes are revealed. Differences in fragment size among alleles represent an RFLP. Thus, the present invention further

5 provides a means to follow segregation of a gene or nucleic acid of the present invention as well as chromosomal sequences genetically linked to these genes or nucleic acids using such techniques as RFLP analysis. Linked chromosomal sequences are within 50 centiMorgans (cM), often within 40 or 30 cM, preferably within 20 or 10 cM, more preferably within 5, 3, 2, or 1 cM of a gene of the present invention.

- 10 In the present invention, the nucleic acid probes employed for molecular marker mapping of plant nuclear genomes selectively hybridize, under selective hybridization conditions, to a gene encoding a polynucleotide of the present invention. In one embodiment, the probes are selected from polynucleotides of the present invention. Typically, these probes are cDNA probes or *Pst I* genomic clones. The length of the
- 15 probes is discussed in greater detail, *supra*, but are typically at least 15 bases in length, more preferably at least 20, 25, 30, 35, 40, or 50 bases in length. Generally, however, the probes are less than about 1 kilobase in length. Preferably, the probes are single copy probes that hybridize to a unique locus in a haploid chromosome complement. Some exemplary restriction enzymes employed in RFLP mapping are EcoRI, EcoRv, and SstI.
- 20 As used herein the term "restriction enzyme" includes reference to a composition that recognizes and, alone or in conjunction with another composition, cleaves at a specific nucleotide sequence.

The method of detecting an RFLP comprises the steps of (a) digesting genomic DNA of a plant with a restriction enzyme; (b) hybridizing a nucleic acid probe, under

25 selective hybridization conditions, to a sequence of a polynucleotide of the present of said genomic DNA; (c) detecting therefrom a RFLP. Other methods of differentiating polymorphic (allelic) variants of polynucleotides of the present invention can be had by utilizing molecular marker techniques well known to those of skill in the art including such techniques as: 1) single stranded conformation analysis (SSCA); 2) denaturing

30 gradient gel electrophoresis (DGGE); 3) RNase protection assays; 4) allele-specific oligonucleotides (ASOs); 5) the use of proteins which recognize nucleotide mismatches,

such as the *E. coli* mutS protein; and 6) allele-specific PCR. Other approaches based on the detection of mismatches between the two complementary DNA strands include clamped denaturing gel electrophoresis (CDGE); heteroduplex analysis (HA); and chemical mismatch cleavage (CMC). Thus, the present invention further provides a
5 method of genotyping comprising the steps of contacting, under stringent hybridization conditions, a sample suspected of comprising a polynucleotide of the present invention with a nucleic acid probe. Generally, the sample is a plant sample; preferably, a sample suspected of comprising a maize polynucleotide of the present invention (e.g., gene, mRNA). The nucleic acid probe selectively hybridizes, under stringent conditions, to a
10 subsequence of a polynucleotide of the present invention comprising a polymorphic marker. Selective hybridization of the nucleic acid probe to the polymorphic marker nucleic acid sequence yields a hybridization complex. Detection of the hybridization complex indicates the presence of that polymorphic marker in the sample. In one embodiment, the nucleic acid probe comprises a polynucleotide of the present invention.
15

RNA Profiling

Plants selected on the basis of expression of AFP1 genes can be used to identify additional genes associated with AFP1 expression. For instance, differences in the expression of specific genes between a disease resistance plant and a susceptible plant
20 can be determined using gene expression profiling. Total RNA is analyzed using the gene expression profiling process (GeneCalling®) as described in U.S. Patent No. 5,871,697, herein incorporated by reference.

UTR's and Codon Preference

25 In general, translational efficiency has been found to be regulated by specific sequence elements in the 5' non-coding or untranslated region (5' UTR) of the RNA. Positive sequence motifs include translational initiation consensus sequences (Kozak, *Nucleic Acids Res.* 15:8125 (1987)) and the 7-methylguanosine cap structure (Drummond *et al.*, *Nucleic Acids Res.* 13:7375 (1985)). Negative elements include stable
30 intramolecular 5' UTR stem-loop structures (Muesing *et al.*, *Cell* 48:691 (1987)) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5'

UTR (Kozak, *supra*, Rao *et al.*, *Mol. and Cell. Biol.* 8:284 (1988)). Accordingly, the present invention provides 5' and/or 3' UTR regions for modulation of translation of heterologous coding sequences.

Further, the polypeptide-encoding segments of the polynucleotides of the present invention can be modified to alter codon usage. Altered codon usage can be employed to alter translational efficiency and/or to optimize the coding sequence for expression in a desired host or to optimize the codon usage in a heterologous sequence for expression in maize. Codon usage in the coding regions of the polynucleotides of the present invention can be analyzed statistically using commercially available software packages such as

5 "Codon Preference" available from the University of Wisconsin Genetics Computer Group (see Devereaux *et al.*, *Nucleic Acids Res.* 12: 387-395 (1984)) or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.). Thus, the present invention provides a codon usage frequency characteristic of the coding region of at least one of the polynucleotides of the present invention. The number of polynucleotides that can be used to determine a

10 codon usage frequency can be any integer from 1 to the number of polynucleotides of the present invention as provided herein. Optionally, the polynucleotides will be full-length sequences. An exemplary number of sequences for statistical analysis can be at least 1, 5,

15 10, 20, 50, or 100.

20 Sequence Shuffling

The present invention provides methods for sequence shuffling using polynucleotides of the present invention, and compositions resulting therefrom.

Sequence shuffling is described in PCT publication No. 96/19256. See also, Zhang, J.-H., *et al. Proc. Natl. Acad. Sci. USA* 94:4504-4509 (1997). Generally, sequence shuffling provides a means for generating libraries of polynucleotides having a desired characteristic which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides which comprise sequence regions which have substantial sequence identity and can be homologously recombined in vitro or in vivo. The population of sequence-recombined polynucleotides comprises a subpopulation of polynucleotides which possess desired or advantageous characteristics and which can be selected by a suitable selection or

screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be a decreased K_m and/or increased K_{cat} over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled wild-type polynucleotide. The increase in such properties can be at least 110%, 120%, 130%, 140% or at least 150% of the wild-type value.

Generic and Consensus Sequences

Polynucleotides and polypeptides of the present invention further include those having: (a) a generic sequence of at least two homologous polynucleotides or polypeptides, respectively, of the present invention; and, (b) a consensus sequence of at least three homologous polynucleotides or polypeptides, respectively, of the present invention. The generic sequence of the present invention comprises each species of polypeptide or polynucleotide embraced by the generic polypeptide or polynucleotide, sequence, respectively. The individual species encompassed by a polynucleotide having an amino acid or nucleic acid consensus sequence can be used to generate antibodies or produce nucleic acid probes or primers to screen for homologs in other species, genera, families, orders, classes, phylums, or kingdoms. For example, a polynucleotide having a consensus sequence from a gene family of *Zea mays* can be used to generate antibody or nucleic acid probes or primers to other *Gramineae* species such as wheat, rice, or sorghum. Alternatively, a polynucleotide having a consensus sequence generated from orthologous genes can be used to identify or isolate orthologs of other taxa. Typically, a polynucleotide having a consensus sequence will be at least 9, 10, 15, 20, 25, 30, or 40 amino acids in length, or 20, 30, 40, 50, 100, or 150 nucleotides in length. As those of skill in the art are aware, a conservative amino acid substitution can be used for amino acids which differ amongst aligned sequence but are from the same conservative

substitution group as discussed above. Optionally, no more than 1 or 2 conservative amino acids are substituted for each 10 amino acid length of consensus sequence.

Similar sequences used for generation of a consensus or generic sequence include any number and combination of allelic variants of the same gene, orthologous, or paralogous sequences as provided herein. Optionally, similar sequences used in generating a consensus or generic sequence are identified using the BLAST algorithm's smallest sum probability (P(N)). Various suppliers of sequence-analysis software are listed in chapter 7 of *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (Supplement 30). A polynucleotide sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, or 0.001, and most preferably less than about 0.0001, or 0.00001. Similar polynucleotides can be aligned and a consensus or generic sequence generated using multiple sequence alignment software available from a number of commercial suppliers such as the Genetics Computer Group's (Madison, WI) PILEUP software, Vector NTI's (North Bethesda, MD) ALIGNX, or Genecode's (Ann Arbor, MI) SEQUENCHER. Conveniently, default parameters of such software can be used to generate consensus or generic sequences.

20

Assays for Compounds that Modulate Function or Expression

The present invention also provides means for identifying compounds that bind to, and/or increase or decrease (i.e., modulate) the function of polypeptides of the present invention. The method comprises contacting a polypeptide of the present invention with a compound whose ability to bind to or modulate the function is to be determined. The polypeptide employed will have at least 20%, preferably at least 30% or 40%, more preferably at least 50% or 60%, and most preferably at least 70% or 80% of the function of the native, full-length polypeptide of the present invention. Generally, the polypeptide will be present in a range sufficient to determine the effect of the compound, typically about 1 nM to 10 µM. Likewise, the compound will be present in a concentration of from about 1 nM to 10 µM. Those of skill will understand that such factors as

concentration, pH, ionic strength, and temperature will be controlled to obtain useful data and determine the presence or absence of a compound that binds or modulates polypeptide function. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be
5 obvious that certain changes and modifications may be practiced within the scope of the appended claims.

The following examples are offered by way of illustration and not by way of limitation.

10

EXPERIMENTAL

EXAMPLE 1

Total RNA Isolation

This example describes the construction of the cDNA libraries.

Total RNA was isolated from corn tissues with TRIzol Reagent (Life Technology
15 Inc. Gaithersburg, MD) using a modification of the guanidine isothiocyanate/acid-phenol procedure described by Chomczynski and Sacchi (Chomczynski, P., and Sacchi, N. *Anal. Biochem.* 162, 156 (1987)). In brief, plant tissue samples were pulverized in liquid nitrogen before the addition of the TRIzol Reagent, and then were further homogenized with a mortar and pestle. Addition of chloroform followed by centrifugation was
20 conducted for separation of an aqueous phase and an organic phase. The total RNA was recovered by precipitation with isopropyl alcohol from the aqueous phase.

Poly(A)+ RNA Isolation

The selection of poly(A)+ RNA from total RNA was performed using PolyATact
25 system (Promega Corporation. Madison, WI). In brief, biotinylated oligo(dT) primers were used to hybridize to the 3' poly(A) tails on mRNA. The hybrids were captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was washed at high stringent condition and eluted by Rnase-free deionized water.

30

cDNA Library Construction

cDNA synthesis was performed and unidirectional cDNA libraries were constructed using the SuperScript Plasmid System (Life Technology Inc. Gaithersburg, MD). The first stand of cDNA was synthesized by priming an oligo(dT) primer containing a Not I site. The reaction was catalyzed by SuperScript Reverse Transcriptase II at 45°C. The second strand of cDNA was labeled with alpha-³²P-dCTP and a portion of the reaction was analyzed by agarose gel electrophoresis to determine cDNA sizes. CDNA molecules smaller than 500 base pairs and unligated adapters were removed by Sephadryl-S400 chromatography. The selected cDNA molecules were ligated into pSPORT1 vector in between of Not I and Sal I sites.

EXAMPLE 2

Sequencing Template Preparation

This example describes cDNA sequencing and library subtraction.

Individual colonies were picked and DNA was prepared either by PCR with M13 forward primers and M13 reverse primers, or by plasmid isolation. All the cDNA clones were sequenced using M13 reverse primers.

Q-bot Subtraction Procedure

cDNA libraries subjected to the subtraction procedure were plated out on 22 x 22 cm² agar plate at density of about 3,000 colonies per plate. The plates were incubated in a 37°C incubator for 12-24 hours. Colonies were picked into 384-well plates by a robot colony picker, Q-bot (GENETIX Limited). These plates were incubated overnight at 37°C.

Once sufficient colonies were picked, they were pinned onto 22 x 22 cm² nylon membranes using Q-bot. Each membrane contained 9,216 colonies or 36,864 colonies. These membranes were placed onto agar plate with appropriate antibiotic. The plates were incubated at 37°C for overnight.

After colonies were recovered on the second day, these filters were placed on filter paper prewetted with denaturing solution for four minutes, then were incubated on top of a boiling water bath for additional four minutes. The filters were then placed on

filter paper prewetted with neutralizing solution for four minutes. After excess solution was removed by placing the filters on dry filter papers for one minute, the colony side of the filters were placed into Proteinase K solution, incubated at 37°C for 40-50 minutes. The filters were placed on dry filter papers to dry overnight. DNA was then cross-linked 5 to nylon membrane by UV light treatment.

Colony hybridization was conducted as described by Sambrook,J., Fritsch, E.F. and Maniatis, T., (in Molecular Cloning: A laboratory Manual, 2nd Edition). The following probes were used in colony hybridization:

First strand cDNA from the same tissue as the library was made from to remove 10 the most redundant clones.

48-192 most redundant cDNA clones from the same library based on previous sequencing data.

192 most redundant cDNA clones in the entire corn sequence database.

A Sal-A20 oligo nucleotide: TCG ACC CAC GCG TCC GAA AAA AAA AAA 15 AAA AAA AAA (SEQ ID NO: 25), removes clones containing a poly A tail but no cDNA.

CDNA clones derived from rRNA.

The image of the autoradiography was scanned into computer and the signal intensity and cold colony addresses of each colony was analyzed. Re-arraying of cold- 20 colonies from 384 well plates to 96 well plates was conducted using Q-bot.

EXAMPLE 3

This example provides an analysis of the anti-fungal polynucleotides of the present invention.

25 A maize disease or stress induced polynucleotide was observed to be highly represented among EST (expressed sequence tags) cDNAs derived from leaf tissue that was either resistant to fungal inoculation or treated with jasmonic acid, a chemical elicitor of plant defense responses. The maize gene is represented by at least five closely related full-length cDNAs contigs (here termed "alleles") that encode either identical or nearly 30 identical peptides. A cDNA for one of these "alleles", named ZmAFP1-1 was sequenced. The other four alleles in maize were sequenced in their coding regions. The ORF for the

gene predicts a small 10 kDa protein rich in histidine, glycine, and aspartic acid, but with a net neutral pI. Protein domain searching revealed homology to a fly (*Sarcophaga peregrina*) antifungal protein of similar molecular weight (Iijima, R. et al., (1993) *J. Biol. Chem.* 268:12055-12061). ClustalW alignment revealed 21-25% overall amino acid 5 identity, with similarity reaching 50%. DNAs for one rice gene and four wheat genes closely homologous to the maize genes were identified, and their full-length coding region sequences were determined. Like the maize gene, the rice and wheat genes were expressed primarily in leaves inoculated with fungal pathogens.

The coding region of ZmAFP1-1 was subcloned into an expression vector that 10 would allow for overexpression of the encoded protein in *E. coli*. In one experiment, the protein was overexpressed as a His-Tag form and purified. (see Expression of Proteins in *Escherichia coli* in Current Protocols in Molecular Biology, eds. Ausubel et al., John Wiley & Sons, 2:16.1.2 (1995)). The purified protein was then assayed against several 15 maize fungal pathogens. The assays did not reveal significant antifungal activity. As one skilled in the art will recognize, expression of each new protein in *E. coli* presents its own unique expression problems (Current Protocols in Molecular Biology, supra). Although not to be limited by theory, there are several possible explanations for why the assays did not reveal significant antifungal activity such as problems with protein folding or 20 incorrect processing of the protein. In addition, there may be problems relating to pathogen specificity of the proteins or the proteins may be indirectly antimicrobial. Screening additional fungal or microbial pathogens could reveal direct antifungal or 25 antimicrobial activity, while a transgenic plant constitutively expressing the AFP1 gene can demonstrate indirect antifungal and antimicrobial activity. Such transgenic plants and their progeny are useful in breeding crop plants with constitutive, hereditary resistance.

EXAMPLE 4

Transformation and Regeneration of Transgenic Plants

Immature maize embryos from greenhouse donor plants are bombarded with a 30 plasmid containing the AFP1 operably linked to a ubiquitin 1 promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to

the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

5 The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

10

Preparation of DNA

A plasmid vector comprising the AFP1 operably linked to a ubiquitin 1 promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl_2 precipitation procedure as follows:

- 100 μl prepared tungsten particles in water
- 10 μl (1 μg) DNA in Tris EDTA buffer (1 μg total DNA)
- 100 μl 2.5 M CaCl_2
- 20 10 μl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multtube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 μl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 μl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-
2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from
5 each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then
transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured
10 every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus
clones are transferred to 288J medium to initiate plant regeneration. Following somatic
embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to
medium for germination and transferred to the lighted culture room. Approximately 7-10
days later, developing plantlets are transferred to 272V hormone-free medium in tubes
15 for 7-10 days until plantlets are well established. Plants are then transferred to inserts in
flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth
chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred
to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored
for disease resistance.

20

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416),
1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0
g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H₂O
25 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to
volume with D-I H₂O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and
cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts
(SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l
thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H₂O
30 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to

volume with D-I H₂O); and 0.85 mg/l silver nitrate and 3.0 mg/l bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 5 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H₂O) (Murashige and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H₂O after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 1.0 mg/l indoleacetic acid and 3.0 mg/l bialaphos (added after 10 sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H₂O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H₂O after adjusting pH to 5.6); and 6 g/l bactoagar 15 (added after bringing to volume with polished D-I H₂O), sterilized and cooled to 60°C.

EXAMPLE 5

Agrobacterium-mediated Transformation

For *Agrobacterium*-mediated transformation of maize with a AFP1, preferably the 20 method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria are capable of transferring the AFP1 nucleotide sequence(s) of interest to at least one cell of at least one of the immature embryos (step 1: 25 the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, 30 the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants

(step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

10

EXAMPLE 6

Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing the AFP1 gene operably linked to a ubiquitin 1 as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

20

Soybean embryogenic suspension cultures can be maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

25

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature (London)* 327:70-73, U.S. Patent No. 4,945,050). A Du Pont Biostatic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

30

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene* 25:179-188), and the 3' region of the

nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the AFP1 gene operably linked to the ubiquitin 1 promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

5 To 50 μ l of a 60 mg/ml 1 μ m gold particle suspension is added (in order): 5 μ l DNA (1 μ g/ μ l), 20 μ l spermidine (0.1 M), and 50 μ l CaCl₂ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μ l 70% ethanol and resuspended in 40 μ l of anhydrous ethanol. The DNA/particle suspension
10 can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are
15 normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

20 Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and
25 inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

30

EXAMPLE 7

Sunflower Meristem Tissue Transformation

Sunflower meristem tissues are transformed with an expression cassette containing the AFP1 gene operably linked to a ubiquitin 1 promoter as follows (see also 5 European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science* 103:199-207). Mature sunflower seed (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled 10 water.

Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer *et al.* (Schrammeijer *et al.* (1990) *Plant Cell Rep.* 9:55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at 15 the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral elements (Murashige *et al.* (1962) *Physiol. Plant.*, 15: 473-497), Shepard's vitamin additions (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops* 20 (University of Minnesota Press, St. Paul, Minnesota), 40 mg/l adenine sulfate, 30 g/l sucrose, 0.5 mg/l 6-benzyl-aminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1 mg/l gibberellic acid (GA₃), pH 5.6, and 8 g/l Phytagar.

The explants are subjected to microprojectile bombardment prior to *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol.* 18:301-313). Thirty to 25 forty explants are placed in a circle at the center of a 60 X 20 mm plate for this treatment. Approximately 4.7 mg of 1.8 mm tungsten microprojectiles are resuspended in 25 ml of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 1.5 ml aliquots are used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000® particle acceleration device.

30 Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains

the AFP1 gene operably linked to the ubiquitin 1 promoter is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet.* 163:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e., *nptII*). Bacteria for plant transformation experiments are grown
5 overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD₆₀₀ of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD₆₀₀ of 0.5 in an inoculation medium comprised of
10 12.5 mM MES pH 5.7, 1 gm/l NH₄Cl, and 0.3 gm/l MgSO₄.

Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators
15 and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on selection and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green,
20 kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assaying for AFP1 activity (see disease resistance assays, above).

NPTII-positive shoots are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot.
30 Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in

soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T₀ plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by AFP1 protein activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T₀

5 plants are identified by AFP1 activity analysis of small portions of dry seed cotyledon.

An alternative sunflower transformation protocol allows the recovery of transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, then rinsed three times with 10 distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter paper moistened with water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-BAP, 0.25 mg/l IAA, 0.1 mg/l GA, and 0.8% Phytagar at pH 5.6) for 24 hours under the dark. The primary leaves are 15 removed to expose the apical meristem, around 40 explants are placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

Approximately 18.8 mg of 1.8 µm tungsten particles are resuspended in 150 µl absolute ethanol. After sonication, 8 µl of it is dropped on the center of the surface of 20 macrocarrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

The plasmid of interest is introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C in a liquid YEP medium (10 g/l yeast extract, 10 g/l Bactopeptone, and 5 25 g/l NaCl, pH 7.0) in the presence of 50 µg/l kanamycin is resuspended in an inoculation medium (12.5 mM 2-mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH₄Cl and 0.3 g/l MgSO₄ at pH 5.7) to reach a final concentration of 4.0 at OD 600. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated 30 on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no BAP, IAA, GA3 and supplemented with 250 µg/ml

cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C incubation conditions.

Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for AFP1 activity using assays known in the art (see above). After positive (i.e., 5 for AFP1 expression) explants are identified, those shoots that fail to exhibit AFP1 activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an 10 additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the initial assay prior to nodal culture.

Recovered shoots positive for AFP1 expression are grafted to Pioneer hybrid 6440 *in vitro*-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and are rinsed three times with distilled water. The sterilized seeds are 20 germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26°C under the dark for three days, then incubated at 16-hour-day culture conditions. The upper portion of selected seedling is removed, a vertical slice is made in each 25 hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

The above examples are provided to illustrate the invention but not to limit its 30 scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications and patent

applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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TECHNICAL DRAWING - SHEET FIVE